AD_____

Award Number: DAMD17-02-1-0397

TITLE: FGF Activation and Signaling in Breast Cancer

PRINCIPAL INVESTIGATOR: Matthew R. Swift

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20007

REPORT DATE: July 2003

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2003	Annual Summary (1 Jul 02-30 Jun 03)

4. TITLE AND SUBTITLE
FGF Activation and Signaling in Breast Cancer

5. FUNDING NUMBERS
DAMD17-02-1-0397

6. AUTHOR(S)
Matthew R. Swift

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Georgetown University
Washington, DC 20007

E-Mail: Mrs8@georgetown.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Fibroblast growth factor binding protein (FGF-BP1) is a crucial molecule that acts to chaperone active FGFs to receptors, thus propagating angiogenic signals for the development of new vasculature. We have shown that FGF-BP1 is expressed in head and neck, skin, cervical, and lung squamous cell carcinomas. A second family member, FGF-BP2 has been identified in our lab and is present in mammary tissue. In this grant, we hypothesized that FGF-BP2 acts in a similar pro-angiogenic capacity as FGF-BP1. The aims were 1) to produce recombinant FGF-BP2 and test its effect on signal transduction, and 2) to study the expression of FGF-BP2 during mouse mammary gland development and carcinogenesis. To date, we have isolated human FGF-BP2 cDNA and protein and confirmed its ability to modulate FGF2. However, we have yet to discover the murine homologue to FGF-BP2. We have identified a third family member, FGF-BP3 in human and mouse. Accordingly, we have adjusted our focus to the characterization of FGF-BP3 activity and expression while continuing our search for murine FGF-BP2. FGF-BP3 has been shown to complex with FGF2, promote increased proliferation, MAPK activation, and anchorage-independent growth in SW-13 adrenal carcinoma cells, and is present in high levels in mouse embryonic tissue.

14. SUBJECT TERMS
Growth factors, signal transduction, in situ hybridization, immunohisto-chemistry, angiogenesis

15. NUMBER OF PAGES
16

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introductions

Tumor growth is dependent upon local-acting growth factors to stimulate the infiltration and growth of new blood vessels from surrounding normal tissue into the tumor mass, a process known as angiogenesis [1,2]. An important class of growth factors that positively regulate angiogenesis is the fibroblast growth factor (FGF) family [3]. Two key members of this family, FGF-1 and FGF-2, require a chaperone molecule to release these factors from extracellular storage and present them to target receptors. These chaperone molecules are known as FGF-binding proteins (FGF-BPs). Two members of the FGF-BP family have been identified so far, FGF-BP1 and FGF-BP2 [4,5]. Previous work from our laboratory has shown that FGF-BP1 can positively modulate the biological activity of FGFs, can support tumor growth and angiogenesis in FGF-2 expressing cell lines, is highly expressed in multiple cancers, and can act as an angiogenic switch *in vivo* in colon cancer cells and SCC. [6,7]. Preliminary data from our lab suggests that FGF-BP2 may act in a similar fashion to chaperone FGFs but has shown distinct expression patterns in tumor samples, particular in human breast cancer samples. A crucial aspect to our study was examination of the expression of FGF-BP2 during mouse development and carcinogenesis, but to date, no murine homologue for FGF-BP2 has been found. Interestingly, a third FGF-BP family member, FGF-BP3 has recently been identified in our lab and a murine homologue for FGF-BP3 was also found. In this study, we began to examine the biological effects of FGF-BP2 but having found mouse and human FGF-BP3, we have refocused our study to explore the effects of this new family member.

Body

We have previously found that FGF-BP2 possesses a unique expression pattern in tissue samples and that the two FGF-BP family members share similar protein identity. Additionally, transfection of FGF-BP2 in SW-13 cells enhances FGF-2 activity, colony formation, and promotes tumor growth in mice. In an effort to further study the tissue expression pattern of FGF-BP2, our goals outlined in Aim 2 were contingent upon the isolation of a murine homologue to FGF-BP2. We had found of one candidate FGF-BP2 EST from mouse based on homology searches with the human and chicken FGF-BP2 gene. Further analysis proved this identification to be incorrect, although this EST did share a high level of homology with both FGF-BP1 and FGF-BP2. Continued genome data base analysis identified a previously unknown human FGF-BP family member isolated from neuroblastoma cell lines. We have named this FGF-BP3 (**Fig 1**). Comparative analysis between human FGF-BP3 and the previously identified EST indicated that this was the murine homologue of FGF-BP3, rather than FGF-BP2 (**Fig 2**). Accordingly, our research aims has been adjusted to investigate the activity of FGF-BP3 as previously described for FGF-BP2. Although we have progressed with the examination of FGF-BP3, our efforts to identify and isolate a murine homologue to FGF-BP2 will continue.

The initial goals stated in Aim 1 have now been modified to reflect the characterization of the novel FGF-BP family member, FGF-BP3. In a similar manner to which our lab first studied FGF-BP1 and FGF-BP2, FGF-BP3 was isolated, amplified, and expressed in a pcDNA3.1 vector construct containing both a V5 and poly-histidine carboxy-terminal tag. SW-13 cells engineered to express FGF-BP3 exhibited an increased level of proliferation with a concurrent increase in MAPK phosphorylation

when compared to parental SW-13 cells (**Fig 3**). Similarly, SW-13/FGF-BP3 cells showed increased levels of anchorage-independent growth over control as determined through colony formation. A neutralizing antibody towards FGF2 eliminated the increase showing this effect to be FGF2-dependent (**Fig 4**). Immunoprecipitation assays using an antibody towards the V5 tag and subsequent immunoblot analysis with an antibody towards FGF2 showed that the proteins form a complex indicating binding between FGF-BP3 and FGF2. Immunoblot analysis using an antibody towards FGF-receptor 1 (FGFR1) indicates that the receptor is also a part of this complex (**Fig 5**). Investigation with migration and invasion assays will be performed to further characterize the biological response of increased FGF-BP3 expression upon FGF2-dependent activity. Additionally, cell-free binding assays will be performed to determine the affinity of FGF-BP3 for FGF1 and FGF2. In keeping with the initial goals of Aim 1, we will also continue to investigate the role of FGF-BP3 in signal transduction pathways, specifically those that are pro-angiogenic. **Preliminary data suggests that FGF-BP3 can mimic the ability of FGF-BP1 and FGF-BP2 to bind FGF2 in SW-13 cells and can enhance FGF2-dependent biological activity in these cells.**

The goals of Aim 2 have essentially remained intact but now focus on the expression of FGF-BP3 mRNA during mouse development. Murine FGF-BP3 was isolated and amplified from N1E-115 mouse neuroblastoma cells. After the PCR product was sequence confirmed, it was used to generate an *in situ* hybridization probe to examine FGF-BP3 expression in embryonic tissue sections. Preliminary experiments with appropriate controls indicate a ubiquitous FGF-BP3 mRNA staining pattern in 11-day old mouse embryo sections. This investigation will be expanded to include murine tissues at different stages of embryonic development as well as adult tissue sections.

Furthermore, a human FGF-BP3 *in situ* hybridization probe has been generated and normal and cancer tissue sections will be stained in order to prepare a clear tissue expression profile for FGF-BP3 in human tissue. Finally, we hope to develop an FGF-BP3 antibody using an FGF-BP3/GST-fusion protein. This will allow us to detect FGF-BP3 protein by immunohistochemistry in both mouse and human tissues sections. In total, this data will help determine the biological role and activity of FGF-BP3 in normal development and carcinogenesis.

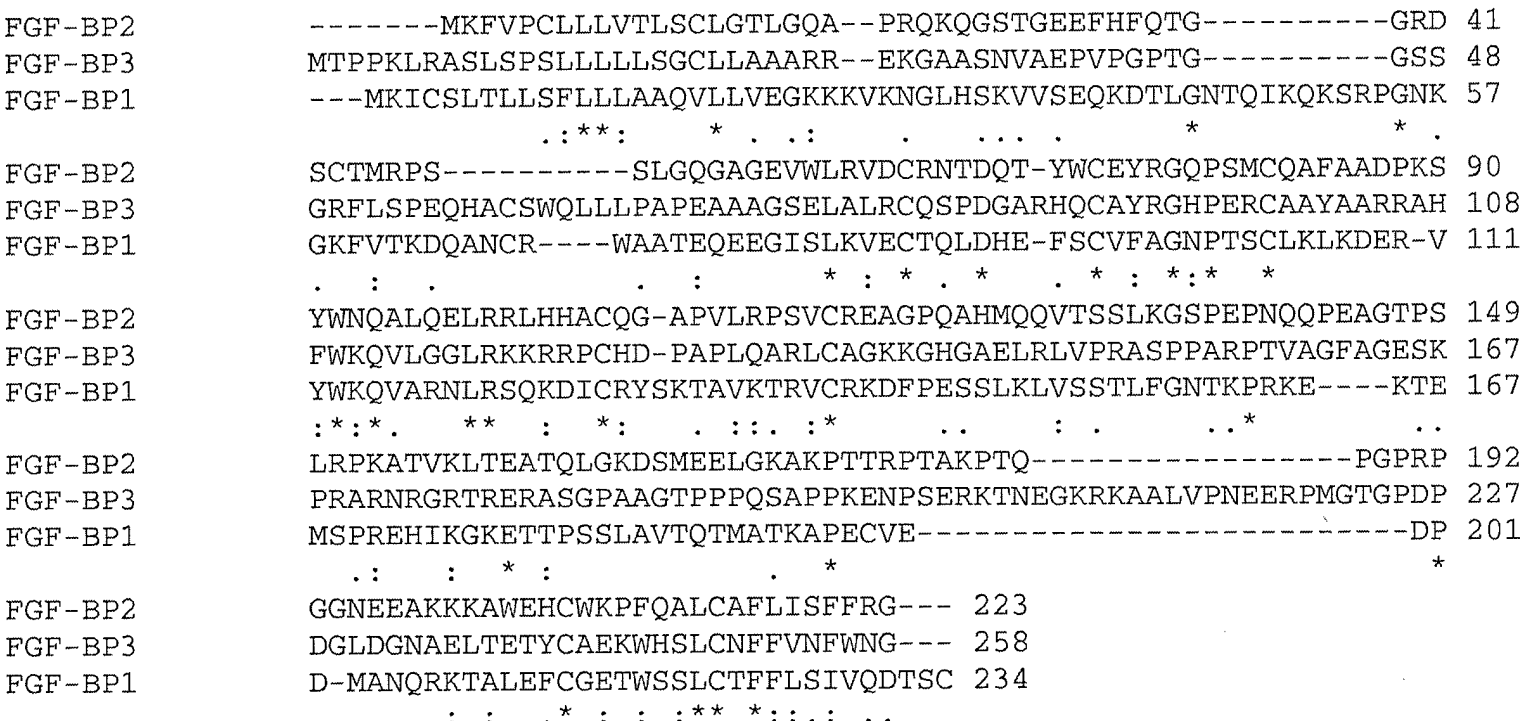


Figure 1: **The FGF-BP3 Family.** Alignment of amino acid sequence of FGF-BP1, FGF-BP2, and an uncharacterized EST on chromosome 10 herein described as a novel family member, FGF-BP3.

A.

```
mBP3        MANAGMSPPRPRASLSPLTLLLLLGGCLLSAAGRDKGAAGREVTRASRPTVGSSGRFVSP 60
hBP3        -----MTPPKLRASLSP-SLLLLLSGCLLAAARREKGAASNVAEPVPGPTGGSSGRFLSP 54
                 *:**: ****** :******.****:** *:****.. .  .. ** ******:**

mBP3        EQHACSWQLLVPAPGTPTGGELALRCQTPGGASLHCAYRGHPERCAATGARRAHYWRRLL 120
hBP3        EQHACSWQLLLPAPEAAAGSELALRCQSPDGARHQCAYRGHPERCAAYAARRAHFWKQVL 114
            **********:*** :.:*.*******:*.**  :************ .*****:*:::*

mBP3        GALRRRPRPCLDPAPLPPRLCA-RKTAGSDLHSPAHPSLPARPSEP-------PRSRARS 172
hBP3        GGLRKKRRPCHDPAPLQARLCAGKKGHGAELRLVPRASPPARPTVAGFAGESKPRARNRG 174
            *.**:: *** ***** .**** :*  *::*:  .:.* ****: .       **:* *.

mBP3        PARSRQS---VRSP---SSQPEKKPLLVKSNSGGRKAGSDPVPEPPAAAGFQPNGLDQNA 226
hBP3        RTRERASGPAAGTPPPQSAPPKENPSERKTNEGKRKAALVPNEERPMGTGPDPDGLDGNA 234
             :*.* *   . :*   *: *:::*   *:*.* ***.  *  * * .:* :*:*** **

mBP3        ELTETYCTEKWHSLCNFFVNFWNG 250
hBP3        ELTETYCAEKWHSLCNFFVNFWNG 258
            *******:*****************
```

B.

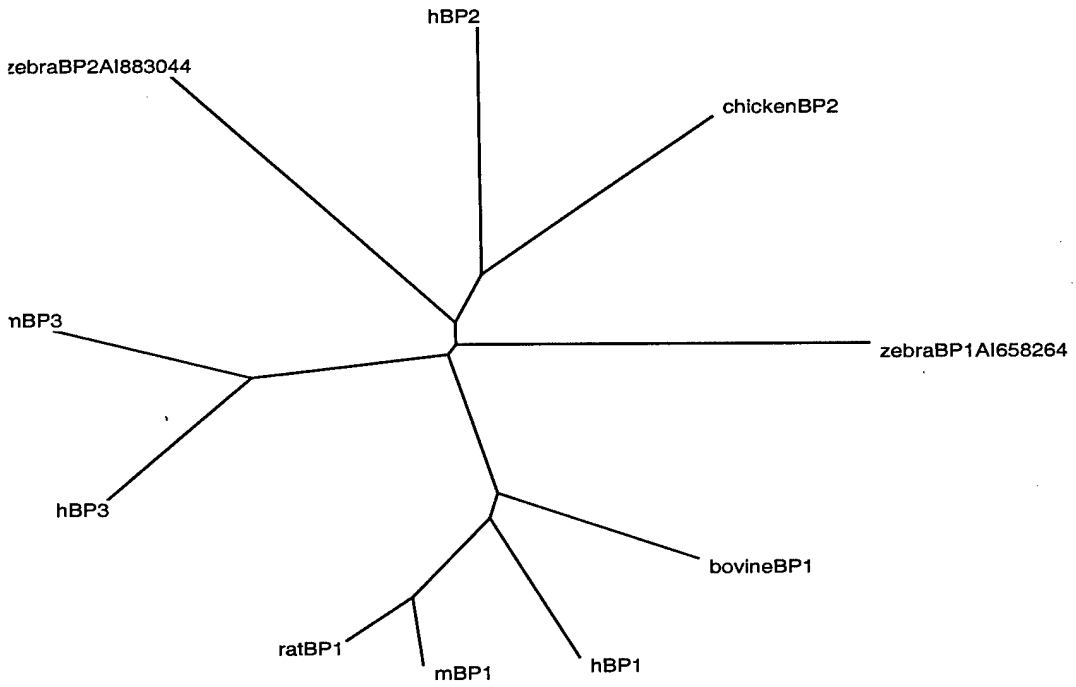
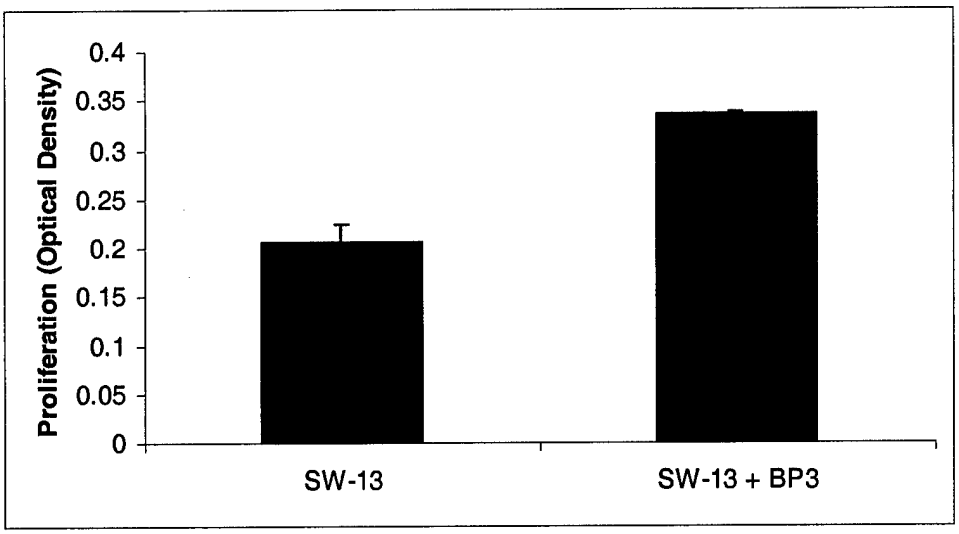


Figure 2: **Human and murine FGF-BP3.** A.) Alignment of amino acid sequence of human FGF-BP3 and a mouse EST determined to be the murine homologue of FGF-BP3. B.) Radial tree diagram demonstrating species homology of the three known members of the FGF-BP family

A.



B.

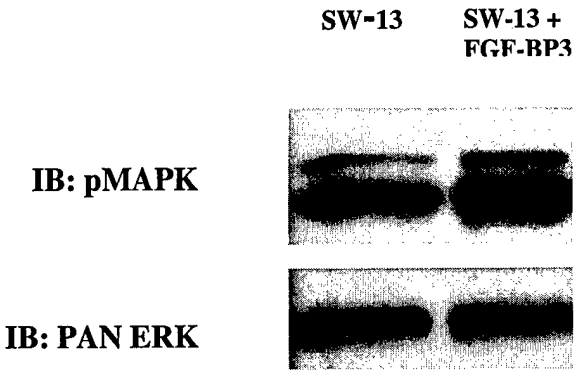


Figure 3: **FGF-BP3 can modulate FGF2-dependant proliferation.** A.) WST-1 proliferation measuring cell viability in parental SW-13 and FGF-BP3-expressing SW-13 cells grown 48 hours in serum-free conditions. B.) MAPK phosphorylation and total ERK expression levels as measured in parental SW-13 and FGF-BP3-expressing SW-13 cells grown overnight in serum-free conditions as measured through immunoblot analysis.

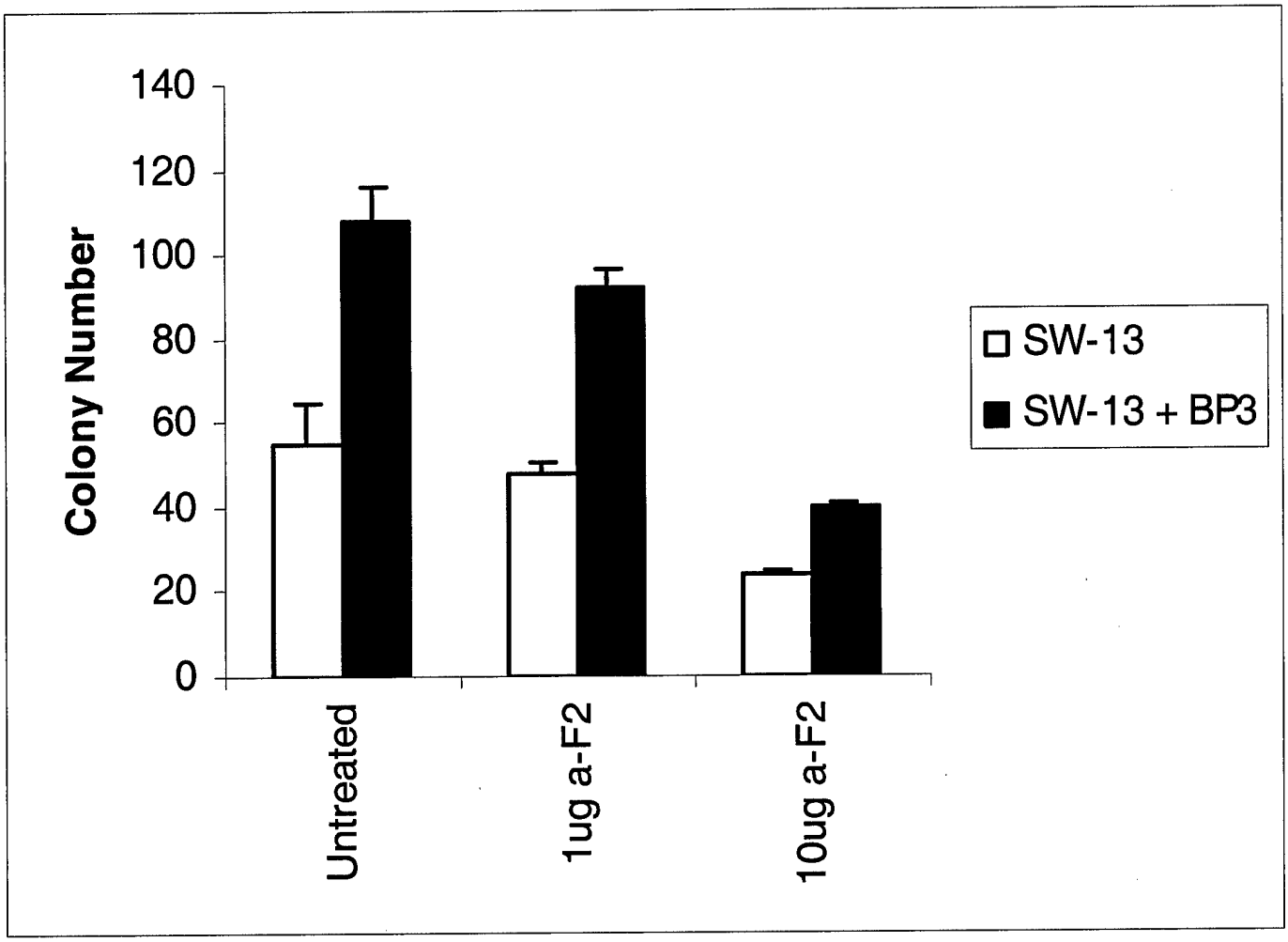


Figure 4: **FGF-BP3 can modulate FGF2-dependant growth.** Soft agar assay measuring anchorage-independent growth in parental SW-13 and FGF-BP3-expressing SW-13 cells grown in both the absence and presence of two doses of a neutralizing antibody to FGF-2. Colonies more than 80 μm in diameter were counted after 14 days of incubation

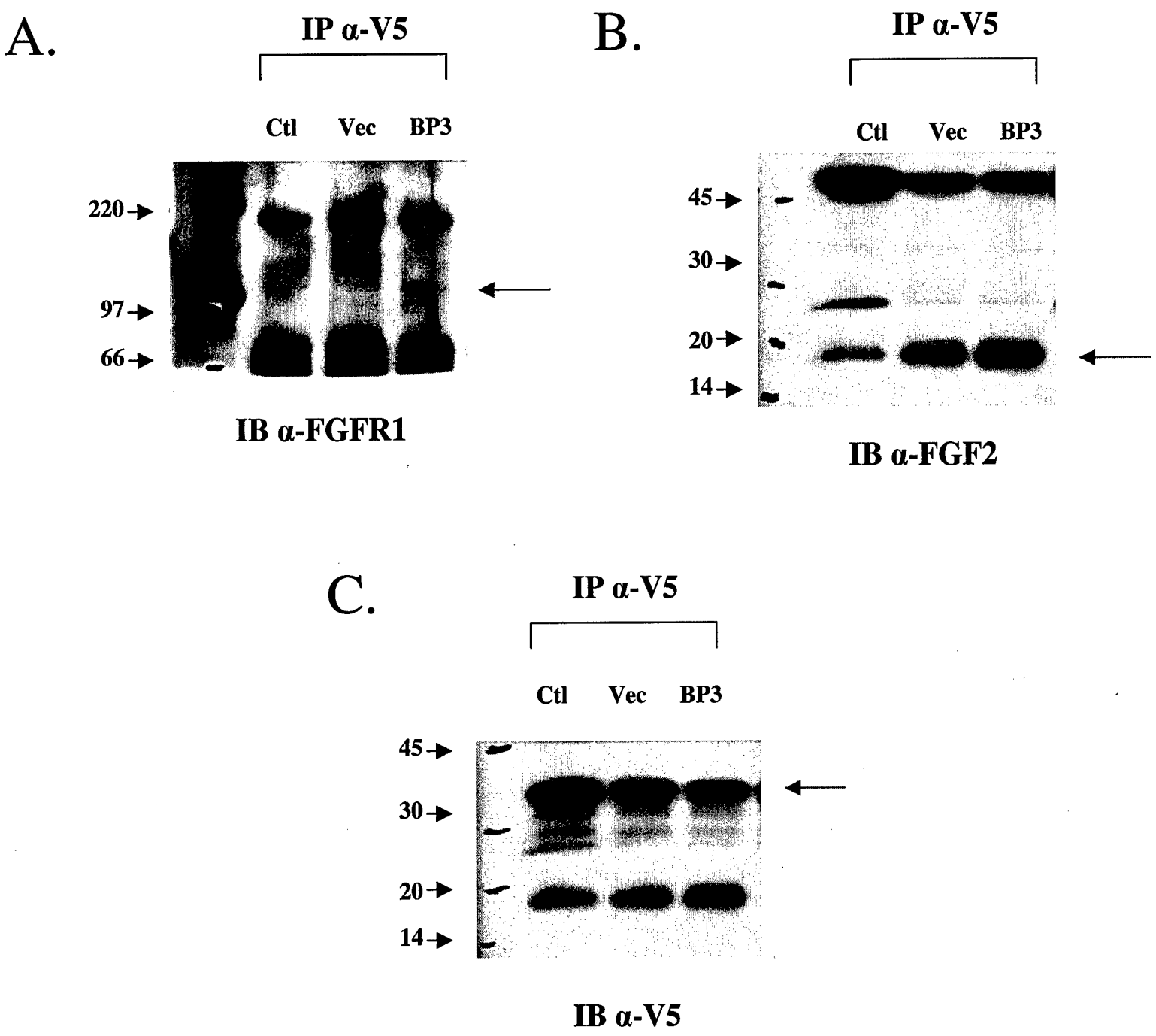


Figure 5: **Immunoprecipitation analysis of the FGF-BP3/FGF2/FGFR1 complex**. Whole cell lysate from parental and pcDNA3.1- or FGF-BP3-expressing SW-13 cells were immunoprecipitated overnight with an antibody against the V5 epitope and immunoblot analysis was performed using antibodies against A.) FGFR1, B.) FGF2, and C.) V5 epitope. Arrows indicate appropriate protein size. Note that basal levels of FGF2 are high in control cells.

Key Research Accomplishments

- Identification of a novel Fibroblast Growth Factor Binding Protein, FGF-BP3
    - We have isolated and amplified human FGF-BP3
    - We have generated vector constructs containing the open reading frame of FGF-BP3 and created stable transfected cells lines expressing FGF-BP3. These SW-13 cells lines exhibit increased proliferation, MAPK phosphorylation, and anchorage-independent growth over control SW-13 cells.
    - We have demonstrated through immunoprecipitation assays that FGF-BP3 forms a complex with both FGF2 and FGFR1 *in vivo*.
    - We have isolated and amplified murine FGF-BP3 from the mouse neuroblastoma cell line, N1E-115.
    - We have generated an in situ hybridization probe for human and murine FGF-BP3 and preliminary results suggest ubiquitous staining for murine FGF-BP3 mRNA in 11-day mouse embryo sections

Conclusions

The goal of **Aim 2, experimental series #1** was to clone murine FGF-BP2 cDNA for *in situ* hybridization studies on mouse mammary gland development and carcinogenesis. The mouse EST sequence we initially thought to include FGF-BP2 was later determined to include the murine homologue to a novel FGF-BP family member, FGF-BP3. All attempts to find mouse FGF-BP2 through genome database analysis have proven to be unsuccessful. Due to the discovery of both human and murine FGF-BP3, we have shifted our focus to characterization of this family member while we continue to search for the mouse homologue to FGF-BP2. Using stable transfected SW-13 cell lines, we have determined that overexpression of FGF-BP3 results in an increased level of proliferation, MAPK phosphorylation, and anchorage-independent growth similar to what has been observed with FGF-BP1 and FGF-BP2 in these same cell lines. As suspected, we have also determined that FGF-BP3 forms a complex with FGF2 and FGFR1 in order to modulate FGF2-dependent biological activities. Further attempts to characterize FGF-BP3-dependent activity in cells are underway. Preliminary *in situ* hybridization experiments with a murine FGF-BP3 probe in 11-day mouse embryo sections have shown a ubiquitous mRNA staining pattern. Additional *in situ* hybridizations involving various stages of murine development as well as adult mouse tissue will be performed to better identify the tissue specificity of murine FGF-BP3. Furthermore, *in situ* hybridization and immunohistochemistry analysis in normal and cancerous human tissue will also help to identify an expression profile for human FGF-BP3.

Abbreviations

FGF: fibroblast growth factor
FGF-BP: fibroblast growth factor binding protein
SCC: squamous cell carcinoma
EST: expressed sequence tag
MAPK: mitogen activated protein kinase

References

1. Folkman J. & Klagsbrun M. (1987) Angiogenic factors. *Science*, **235**, 442.
2. Folkman J. (1986) How is blood vessel growth regulated in normal and neoplastic tissue? G.H.A. Clowes memorial Award lecture. *Cancer Res*, **46**, 467.
3. Baird A. & Klagsbrun M. (1991) The fibroblast growth factor family. *Cancer Cells*, **3**, 239.
4. Wu D.Q., Kan M.K., Sato G.H., Okamoto T. & Sato J.D. (1991) Characterization and molecular cloning of a putative binding protein for heparin-binding growth factors. *J Biol Chem*, **266**, 16778.
5. Powers C.J., McLeskey S.W. & Wellstein A. (2000) Fibroblast growth factors, their receptors and signaling. *Endocr Relat Cancer*, **7**, 165.
6. Czubayko F., Smith R.V., Chung H.C. & Wellstein A. (1994) Tumor growth and angiogenesis induced by a secreted binding protein for fibroblast growth factors. *J Biol Chem*, **269**, 28243.
7. Czubayko F., Liaudet-Coopman E.D., Aigner A., Tuveson A.T., Berchem G.J. & Wellstein A. (1997) A secreted FGF-binding protein can serve as the angiogenic switch in human cancer. *Nat Med*, **3**, 1137.